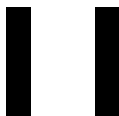


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PCR

Kazuko Aoyagi

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INTRODUCTION

The principle of the polymerase chain reaction (PCR) was first reported in 1971 (Kleppe et al., 1971), but it was only after the discovery of the thermostable Taq DNA polymerase (Saiki et al., 1988; Lawyer et al., 1989) that this technology became easy to use. Initially the thermal cycling was handled manually by transferring samples to be amplified from one water bath to another with the addition of fresh enzyme per cycle after the denaturation step (Saiki et al., 1986; Mullis et al., 1986). Today, 30 years later, we are fortunate to have thermal cyclers, along with enzymes and other reagents dedicated for various PCR applications. The advances in PCR technology and the number of annual publications using PCR in some area of the research has grown tremendously from a single-digit number to 1.6×10^4 in 1999 (Medline search). The popularity of the PCR method lies in its simplicity, which permits even a lay person without a molecular biology degree to run a reaction with minimum training.

However, this easy “entry” can also act as a “trap” to encounter common problems with this technology. The purpose of this chapter is to help you select and optimize the most appropriate PCR strategy, to avoid problems, and to help you think your way out of problems that do arise. While your research topic may be unique, the solutions to most PCR problems are less so. Employing one or a combination of methods mentioned in this chapter could solve problems. I encourage readers to spend time in setting up the lab, choosing the appropriate protocol, optimizing the con-

ditions and analysis method *before* running the first PCR reaction. In the long run, you will save time and resources.

This chapter provides practical guidelines and references to in-depth information. Other useful information is added in the Appendix to help you navigate through various tools available in today's market.

DEVELOPING A PCR STRATEGY:THE PROJECT STAGE

Assess Your Needs

First ask yourself what outcome you need to achieve to feel successful with your experiment (Table 11.1). What kind of information do you need to get? Is it qualitative or quantitative? Are you setting up a routine analysis to run for the next two years, or is this for the manuscript you need to send to the editor in a hurry in order for your paper to get accepted? Your priorities will help you choose the method that best fits your needs.

Table 11.2a shows an example of a list for a researcher who needs to develop a PCR method where approximately 48 genes will be studied for relative gene expression in response to various drug treatments to be given over a three-year period. In contrast, Table 11.2b shows a list of a scientist who wishes to clone a gene with two different mRNA forms generated by alternative splicing

Table 11.1 Priority Check List

Objectives	High/Medium/Low
Quantitative	
Sensitivity	
Fidelity	
High-throughput	
Reproducibility	
Cost-sensitive	
Long PCR product	
Limited available starting material	
Short template size	
Gel based	
Simple method	
Nonradioactivity involved	
Automated	
Long-term project	
DNA PCR	
RNA PCR	
Multiple samples	
Multiplex	

Table 11.2a Priority List: Researcher 1

Objectives	High/Medium/Low
Quantitative	H
Sensitivity	H
Fidelity	M
High-throughput	M
Reproducibility	H
Cost-sensitive	M
Long PCR product	L
Limited available starting material	M
Short template size	H
Gel based	L
Simple method	H
Nonradioactivity involved	H
Automated	H
Long-term project	H
DNA PCR	L
RNA PCR	H
Multiple samples	H
Multiplex	H

Table 11.2b Priority List: Researcher 2

Objectives	High/Medium/Low
Quantitative	M
Sensitivity	M
Fidelity	H
High-throughput	L
Reproducibility	H
Cost-sensitive	H
Long PCR product	L
Limited available starting material	M
Short template size	M
Gel based	H
Simple method	L
Nonradioactivity involved	L
Automated	L
Long-term project	L
DNA PCR	L
RNA PCR	H
Multiple samples	L
Multiplex	L

mechanisms. The purpose of Researcher 2 is to amplify the cDNA and to demonstrate the size difference by separating the two forms by gel electrophoresis. The data are needed for a manuscript due in two months. You can see the differences between the priorities and needs of the two researchers.

After setting clear objectives of what your PCR reaction must accomplish, check that you have the adequate resources. This includes not only budget but also head count, skill level, time, equipment, sequence information, sample supply, and other issues. If time is most critical, then you may require a colleague's assistance or a new instrument to do the project as quickly as possible. In a similar token, if the sample is difficult to obtain in abundance, the choice of PCR that minimizes the sample requirement becomes more important.

Selecting one PCR strategy that optimally satisfies every research need is unlikely. At this early planning stage, a compromise between competing needs will likely be required. Remember that after all the planning is complete, the final PCR strategy still has to evolve at the lab bench.

Identify Any Weak Links in Your PCR Strategy

There are many parameters that affect the outcome of a PCR reaction. Some examples are as follows:

- PCR reaction chemistry (enzyme, nucleotide, sample, primer, buffer, additives).
- PCR instrument type (ramp time, well-to-well homogeneity, capacity to handle many samples).
- Thermal cycling conditions (two-step, three-step, cycle segment length—i.e., denaturation, annealing, and extension—ramp time, etc.).
- Sample collection, preparation, and storage (DNA, RNA, microdissected tissue, cells, and archived material).
- PCR primer design.
- Detection method (simultaneous detection, post PCR detection).
- Analysis method (statistical analysis).

Like the weakest link in a chain, your final result will be limited by the parameter that is least optimum. For example, suppose that you're studying the tissue-specific regulation of two mRNA forms. Regardless of the time spent optimizing the PCR reaction, instrument type, and everything to near-perfection, the use of agarose gel electrophoresis may not allow you to reach the conclusion that there are two different mRNA forms if their molecular weights are similar. You might require a separation technique with greater resolving power.

Suppose that your objective requires quantitative PCR. RNA from 30 samples is collected and RT-PCR is performed. The PCR reaction is run in duplicate and repeated twice on two different

days. One-step RT-PCR is done using the same RNA samples, and PCR products are analyzed by polyacrylamide gel electrophoresis (PAGE). For some unknown reason, the second experiment shows different quantitative data. Which data are correct? Without a sufficient number of samples to calculate standard deviation, one cannot make any quantitative analysis. For quantitative PCR, the sample size has to be large enough and the standard curve must show that PCR was linear within the range one is examining. To do this, serial dilution of a positive control must be run simultaneously, and the test samples have to fall within this range of amplification. Minimums of three to four samples are required for reliable statistical analysis of the data. It is also a good idea to generate enough cDNA to run multiple experiments to reduce error due to differences in the cDNA synthesis step. The positive control must also be properly stored so that loss or damage of DNA does not generate false negative results.

High-tech, automated PCR synthesis and detection systems are useless if the sample preparation destroys the mRNA, co-purifies PCR inhibitors, or the PCR primer design amplifies genomic DNA. Your results will only be as good as the weakest parameter in your PCR strategy.

Manipulate the Reaction to Meet Your Needs

Table 11.3 describes positive and negative effectors of the PCR reaction. These data can help you plan your experiment or modify your strategy if your results aren't satisfactory.

DEVELOPING A PCR STRATEGY: THE EXPERIMENTAL STAGE

What Are the Practical Criteria for Evaluating a DNA Polymerase for Use in PCR?

An appreciation of what your research objective requires from a PCR product should be central to your selection of a thermostable DNA polymerase. Were you planning to identify a rare mutation in a heterogeneous population as in allelic polymorphisms (Frohman, Dush, and Martin, 1988)? As the copy number gets smaller (less than 10), the need for high-fidelity enzyme or enzyme mixes increases, as discussed below. In contrast, if you're screening a batch of transgenic mice for the presence or absence of a marker gene via Southern hybridization, enzyme fidelity might not be as crucial. Most applications do not require high

Table 11.3 Positive and Negative Effectors of a PCR Reaction

To Enhance This Parameter	Manipulate One or More of These Components	
Fidelity and specificity	Enzyme	Select an enzyme with potent 3'–5' Exonuclease activity.
	Primer design	Include mismatches at 3' end, which can help discriminate against homologous sequences such as pseudogenes. Enzyme selection can enhance this effect. With Taq polymerase, relative amplification efficiencies with 3'-terminal mismatches is greater for A:G and C:C than for other nucleotide pairs (Kwok et al., 1995). Use longer primers (refer to section “What Are the Steps to Good Primer Design?”). Primers less than 15 nucleotides do not give enough specificity from a statistical point of view.
	PCR cycling condition	Increase annealing temperature. Reduce cycle segment time (denaturation, annealing, etc.). Lower cycling number.
	Reaction chemistry	Decrease $[Mg^{2+}]$. Apply a hot start strategy (Erlich, Gelfand, and Sninsky, 1991). Check that concentration and pH of dNTP solution(s) is correct. Decrease primer concentration.
	Template	Confirm that template is intact, not nicked, and free of contaminants and inhibitors. Confirm the presence of sufficient starting copy number.
	Method of analysis	Minimize contamination and handling errors; use an automated analysis system. Use sufficient sample number to enable reliable statistical analysis. Check for erroneous manipulation (pipetting errors, etc.).
	Clean lab practice	Use a positive displacement pipette. Use a separate room to set up experiments. Wear gloves. Use UNG and dUTP (Longo, Berninger, and Hartley, 1990).
	Cycler	Check that the temperature profile is consistent at every position in the heating block. Decrease ramp time. Check for tight fit between reaction vessels and heating block.
Efficiency of doubling/cycle	Reaction	Increase concentration of dNTPs and enzymes. Use minimal concentrations of DMSO, DMF, formamide, SDS, gelatin, glycerol (see Table 11.7).

Table 11.3 (Continued)

To Enhance This Parameter	Manipulate One or More of These Components	
	Template	Confirm that template is unnicked, free of contaminants and inhibitors. Use a smaller size template DNA (get more molecules per pg of input template, and less complexity for primer annealing). For example, PCR product vs. genomic DNA. Decrease amplicon size.
	Enzymes	Taq > Pfu, >>Stoffel fragment.
	Cycling	Decrease cycling time or use a shuttle profile (Cha et al., 1992). Decrease the size of the reaction tube. Check for tight fit between reaction vessels and heating block.
	Cycler	Decrease ramp time.
	Primer design	Use forward and reverse primers that have similar length and GC content. Confirm that primers do not form primer-dimer or hairpin structure.
Reproducibility	Sample	Ensure that template is clean and intact. Confirm presence of sufficient starting template and sufficient sample number for statistical analysis.
	Reagents	Use the same lots of primer and buffers between experiments. Store enzyme in small aliquots. Investigate for presence of contaminating template and inhibitors to PCR reaction.
	Controls	Include positive and negative controls with all experiments.
	Cycling	Use a hot-start strategy (Kellogg et al., 1994). Use the same cycler between experiments.
Quantitative	Template	Confirm the quantity of the template. Confirm template preparation is clean. Investigate for presence of contaminating template and inhibitors to PCR reaction.
	Experimental design	Include triplicate or quadruplicate samples. Use a statistically sufficient number of samples. Prepare a standard curve to demonstrate the range over which PCR product yield provides a reliable measure of the template input. Robust: Confirm that chemistry, primer design, tubes, thermal cycler, and other factors are optimized.
	Analysis	Confirm the analytical method's accuracy/resolution. Is it accurate during the exponential phase of PCR?

Table 11.3 (Continued)

To Enhance This Parameter	Manipulate One or More of These Components	
High-throughput		Use appropriate controls. Repeat experiments when data are outside of standard deviation limits. Minimize the manipulations from start to finish.
	Cycler	Check that the temperature profile is consistent at every position in the heating block.
	Control	Confirm that controls have similar sequence profile and amplification efficiency. Confirm that PCR was linear by producing a standard curve.
	Analysis	Use an automated system to reduce handling steps.
	Detection	Check the detection strategy's sensitivity and ability to measure yield in the exponential phase of PCR. Confirm that the technique has high sensitivity and magnitude over a wide dynamic range.
	Instrument	Select a system that handles microtiter plates and multiple sample simultaneously.
	Reaction	Use a hot-start PCR strategy (D'Aquila et al., 1991; Chous et al., 1992; Kellogg et al., 1994). Use a master PCR reagent mix. Use aliquots taken from the same lot of material; don't mix aliquots from different lots.
	Sample preparation	Use of robotics. Storage of sample as cDNA or ethanol precipitate, rather than RNA in solution.
	Cycling	Use one cycling strategy for all samples. Decrease the cycling time.
	Analysis	Use an automated system.
Sensitivity	Detection	Use an automated detection system to monitor the exponential phase.
	Detection	Monitor specific PCR product formation by hybridization via nucleic acid probe. Use fluorescent intercalating dye (Wittwer et al., 1997).
	Reaction	Use a nested PCR strategy (Simmonds et al., 1990). Note: Sensitivity is gained at the expense of quantitation. Use a hot-start PCR strategy. Use UNG and dUTP to prevent carryover.
	Analysis	Use a real time PCR strategy that detects low levels of amplicon missed by gel

Table 11.3 (Continued)
To Enhance This
Parameter

Manipulate One or More of These Components	
	electrophoresis. When hybridization probes are used, primer-dimer formation will not mask the authentic product, even after 40 cycles. This is not true for SYBR® Green or Amplifluor.
Control	Nested PCR or extra manipulation may be needed for other non-real-time PCR based techniques. “Hot” nested PCR is one such example that elegantly combines the qualities of nested PCR with the high resolution of PAGE (Jackson, Hayden, and Quirke, 1991). Include positive and negative controls; when the target is not detected, one can conclude that target was below 100 copies, etc., which makes the data more meaningful than just saying it was not detected.
Lab setup	Clean lab. No contamination.
Experimental design	Check primer design. If amplifying related genes is a concern, design the primer to create mismatches at the 3’ end using the most heterogeneous sequence region.

fidelity, but one needs to be aware when high fidelity has to be considered. During planning, one should also consider the many ways a PCR reaction can be manipulated to achieve a given end, as discussed throughout this chapter.

The data in Table 11.4 are provided to highlight the biochemical properties of common PCR-related enzymes and help you develop a selection strategy. For a comprehensive comparison of thermostable DNA polymerases, see Perler, Kumar, and Kong (1996), Innis et al. (1999), and Hogrefe (2000). However, biochemical data and logic can’t always predict the most appropriate enzyme for PCR; experimentation might still be required to determine which enzyme works best. Abu Al-Soud and Radstrom (1998) demonstrate that contaminants inhibitory to PCR vary with the sample source, and that experimentation is required to determine which thermostable DNA polymerase will produce successful PCR. A second illustration of the difficulty in predicting success based on enzymatic properties are the Archae DNA polymerases, which have not become premiere PCR enzymes despite their extreme thermostability and good proofreading activity.

Table 11.4 Selected Properties of Common Thermostable DNA Polymerases

Enzyme	Proofreading (3'–5' exonuclease)	5'–3' Exonuclease	Heat Stability (min before 50% activity remains)	Processivity (dNTP/ binding)	Extension Rate (dNTP/ s/mol)
<i>Taq</i> DNA polymerase	Absent	Present	9 at 97.5°C (40–60 at 95°C, depending on protein concentration ^{a,b})	50–60	60–150
Stoffel fragment	Absent	Absent	21 at 97.5°C	5–10	130
<i>Tth</i> DNA polymerase	Absent	Present			25
<i>rTth</i> XL	Trace	Present		30–40	
AmpliTaq CS	Absent	Absent		50–60	
<i>UITma</i> DNA polymerase	Present (low)	Absent	50 at 97.5°C		
<i>Pfu</i> DNA polymerase (native and recombinant)	Present	Absent	1140 at 95°C ^a	10 ^a	60
<i>Pfu</i> DNA polymerase (exo-form)	Absent	Absent	1140 at 95°C ^a	11 ^a	
(<i>Pyrococcus</i> <i>species</i> GB- D)	Present	Absent	1380 at 95°C ^c		>80
(aka Deep Vent [®])			480 at 100°C		
<i>Tli</i> Pol (aka Vent [®])	Present	Absent	402 at 95°C ^c	7	67
			108 at 100°C		
Herculase enhanced DNA polymerase	Present	Present ^a			
<i>Tbr</i> DNA polymerase (Dynazyme TM) ^e	Absent	Present	150 at 96°C		
Platinum <i>Pfx</i> ^f	Present	Absent	720 at 95°C 180 at 100°C	100–200	100–300
Platinum <i>Taq</i> ^f	Absent	Present	96 at 95°C	50–60	60–150
Advantag Polymerase ^g	Absent	Absent	40 at 95°C		40
<i>Tac</i> Pol	Present	Absent	30 at 75°C		
<i>Mth</i> Pol	Present	Absent ^d	12 at 75°C		
ThermalAce TM <i>Pyolobus</i> <i>fumarius</i> ^h	Present	Absent		5-fold greater than <i>Taq</i> DNA Polymerase	
Hot <i>Tub</i> (<i>T.</i> <i>flaius</i>) ⁱ	Present	Absent	Similar to <i>Taq</i>		

Source: Unless otherwise noted, all data from Perler, Kumar, and Kong (1996).

^a Data provided by H. Hogrefe, Stratagene, Inc.

^b New England Biolabs Catalog, 2000.

^c Z. Kelman (JBC 274:28751); present according to Perler.

^d Data provided by D. Titus, MJ Resesarch, Inc.

^e Data provide by D. Hoekzema, Life Technologies Inc.

^f Data provided by J. Ambroziak, Clonetech Laboratories Inc.

^g Data provided by Invitrogen, Inc.

^h Lawyer et al. (1993), PCR Methods & application pp. 275–286.

ⁱ Data provided by Amersham Pharmacia Biotech, Inc.

Fidelity

Fidelity could be defined as an enzyme's ability to insert the proper nucleotide and eliminate those entered in error. As thoroughly reviewed by Kunkel (1992), fidelity is not a simple matter; there are several steps during the polymerization of DNA where mistakes can be made and corrected. Still most practical discussions of fidelity focus on the proofreading function provided by an enzyme's 3'-5' exonuclease activity. Cline, Braman, and Hogrefe (1996) compared the fidelity of several thermostable DNA polymerases side by side, taking care to optimize the conditions for each enzyme. They observed the following fidelity rates (mutation frequency/bp/duplication), in order: *Pfu* (1.3×10^{-6}) > *Deep Vent* (2.7×10^{-6}) > *Vent* (2.8×10^{-6}) > *Taq* (8.0×10^{-6}) > *exo⁻ Pfu* and *UITma* ($\sim 5 \times 10^{-5}$). These and similar data should be viewed in relative rather than absolute terms, because assay methods affect the absolute number of detected misincorporations (André et al., 1997), and sample source can affect the performance of enzymes differentially and unpredictably (Abu Al-Soud and Radstrom, 1998).

Proofreading activity can also reduce PCR yield, especially in reactions that generate long PCR products. The greater time required to extend the fragment increases the chance of primer degradation by the 3'-5' exonuclease activity (de Noronha and Mullins, 1992 and Skerra, 1992). The problem of reduced yield can be corrected by including an enzyme with strong proofreading activity into a PCR reaction with a polymerase that lacks a strong proofreading activity (Barnes, 1994; Cline, Braman, and Hogrefe, 1996; MJ Research Inc. Application Bulletin, 2000).

Heat Stability

Is a higher reaction temperature always helpful and necessary? No. For most DNA-based PCR, the consensus is that hot-start PCR increases both sensitivity and yield by preventing nonspecific PCR product formation (Faloona et al., 1990). Higher temperatures can melt secondary structures, but there are limitations to the use of heat. Very high denaturation temperatures can also damage DNA, through depurination and subsequent fragmentation, especially during long PCR reactions (Cheng et al., 1994). It can also increase hydrolysis of RNA in one step RT-PCR in the presence of magnesium ions (Brown, 1974). In order to reduce heat-induced damage, incorporation of additives such as DMSO is used (see later section on additives).

Choosing an enzyme with specialized activities will not produce

the desired results unless the appropriate conditions are applied. For example, UITma™ DNA polymerase has a pH optimum for polymerase activity of 8.3 and exonuclease activity at pH 9.3 (Bost et al., 1994). Likewise, presence of metal ions can favor one activity over the other for many polymerases.

Long PCR

Additives such as single-stranded binding protein (Rapley, 1994), T4 gene 32 protein (Schwarz, Hansen-Hagge, and Bartram, 1990), and proprietary commercial products may increase the production efficiency of long PCR. However, fidelity was also shown to be crucial to the replication of large products via PCR (Barnes, 1994). By supplementing PCR reactions containing *Taq* DNA polymerase (which lacks proofreading activity) with proofreading-rich *Pfu* DNA polymerase, Barnes generated fragments up to 35 kb. Bear in mind that proofreading activity can potentially reduce yield, especially with large PCR products. As discussed above, this problem can be avoided by utilizing a combination of polymerases that possess and lack strong proofreading activity.

The availability of specialized, designer enzymes are an attractive strategy that shouldn't be ignored. However, selecting the right enzyme(s) is one step among many, and can't guarantee the desired result. One near-term example is the importance of enzyme concentration. The concentration of polymerase applied to a PCR reaction ranges from one to four units per 100 μ L. Greater concentrations can increase formation of nonspecific PCR products. The importance of optimizing other parameters, such as buffer component, primer design, and cycling conditions is shown in Table 11.3 and Table 11.5.

How Can Nucleotides and Primers Affect a PCR Reaction?

Nucleotide Concentration

The standard concentration of each nucleotide in the final reaction is approximately 200 μ M, which is sufficient to synthesize 12.5 μ g of DNA when half of the dNTPs are incorporated. Adding more nucleotide is unnecessary and detrimental. Too much nucleotide reduces specificity by increasing the error rate of the polymerase and also chelates magnesium, changing the effective optimal magnesium concentration (Gelfand, 1989; Coen, 1995).

Primer Concentration

The standard primer concentration is 100 to 900 nM; too much primer can increase the formation of nonspecific products. It is

Table 11.5 Optimizing MgCl₂ Concentration for PCR

Component	Final Concentration	Per Reaction	1 mM	2 mM	3 mM	4 mM	6 mM	8 mM	10 mM
10× PCR buffer	1×	10 μl	40.0	40.0	40.0	40.0	40.0	40.0	40.0
50 μM forward primer	0.5 μM	1 μl	4.0	4.0	4.0	4.0	4.0	4.0	4.0
50 μM reverse primer	0.5 μM	1 μl	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Template DNA	Optimum	10 μl	40.0	40.0	40.0	40.0	40.0	40.0	40.0
100 mM MgCl ₂	Various	Various	4.0	8.0	12.0	16.0	24.0	32.0	40
25 mM dNTP mix	0.2 mM	0.8 μl	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Taq polymerase 5 U/μl	2.5 U	0.5 μl	2.0	2.0	2.0	2.0	2.0	2.0	2.0
H ₂ O		To 100 ul	To 400 μl	To 400 μl	To 400 μl	To 400 μl	To 400 μl	To 400 μl	To 400 μl

especially important to adjust the primer concentration when the target sequence is rare or the template amount is low. Less primer is needed in these cases; too much primer will generate primer-dimers or smearing of the product visualized by agarose gel electrophoresis. For most applications it is practical to apply the standard concentrations cited above and to focus effort on optimizing other critical parameters. For real-time PCR multiplex applications, it is recommended that a primer matrix study be performed (Table 11.6a,b) to ensure the limiting primer concentration for an endogenous control. This way the target gene amplification is not compromised by competition for reagents in the same reaction tube (well). This recommendation applies to all housekeeping genes regardless of the abundance level (i.e., needed not only for rRNA but also for less abundant genes, e.g., glyceraldehyde 3-phosphate dehydrogenase, cyclophilin, and hypoxanthine-guanine phosphoribosyl-transferase).

The range of final concentration for forward and reverse primers is 100 to 900 nM in the matrix below. Perform an initial series of experiments to find the rough range of an optimum primer concentration. Follow with a second series of experiments to fine-tune the primer concentration range. In the following example, the final results suggest a forward primer concentration

Table 11.6a Primer Matrix Study

	Primer concentration (nM)	Forward Primer			
		100	300	600	900
Reverse Primer	100	++	+	—	----
	300	+	+	—	----
	600	—	---	---	----
	900	--	---	----	----

Table 11.6b Primer Matrix Study: Final Primer Optimization Matrix

	Primer concentration (nM)	Forward Primer					
		100	120	140	160	180	200
Reverse Primer	100	+	+	+	++	++	++
	120	+	+	+	++	++	++
	140	+	+	++	++	++	+++
	160	+	+	+	+	+	++
	180	—	—	—	—	—	—
	200	----	--	—	—	—	—

of 200 nM and reverse primer at 140 nM. Both the specificity and the yield can be scored for excellent (+++), good (++), fair (+), and similarly for poor (—), very bad (---) based on no signal, smear, and low yield.

Nucleotide Quality

The benefits of using extremely pure solution nucleotides as compared to standard lyophilized nucleotides include proper pH and absence of nuclease. A nucleotide solution at too low or high a pH can shift the overall pH of the reaction buffer and decrease yield, as can unequal quantities of the four nucleotides. The proper quantitation and pH adjustment of nucleotide solutions is discussed in Chapter 10, “Nucleotides, Oligonucleotides, and Polynucleotides.”

How do the Components of a Typical PCR Reaction Buffer Affect the Reaction?

The buffer impacts the amplification by maintaining pH range, minimizing effect of inhibitors, protecting enzymes from premature loss of activity, stabilizing template, and more. Because polymerases have a narrow optimum pH range, a slight shift of pH, as little as 0.5 to 1 can reduce the yield of the PCR products. Because

Tris buffer changes its pH with temperature, it is not an ideal buffer for Taq polymerase. Table 11.7 summarizes the effects of several common additives on Taq polymerase. Their impact and optimum concentrations might differ for other enzymes, but the data regarding Taq polymerase is a starting point. Consult the manufacturers of other enzymes for more details.

Magnesium

The concentration of MgCl_2 affects enzyme specificity and reaction yield. In general, lower concentrations of Mg^{2+} leads to specific amplification and the higher concentration encourages nonspecific amplification. The effective concentration of Mg^{2+} is dependent on the dNTP concentration as well as the template DNA concentration and primer concentration. The strategy illustrated in Table 11.5 can be used to optimize Mg^{2+} concentration as well as other additives described below.

Additives and Contaminants

Detergent, gelatin, and other components are often included to reduce the negative effect of contaminants (Gelfand, 1992) (Table 11.7). Tween eliminates the effects of SDS, which can be carried over from sample preparation. Detergent can also stabilize the activity of some enzymes, such as Taq polymerase. When the amount of template is very small, nuclease can degrade the precious DNA, but the presence of “carrier” DNA can prevent this. Gelatin helps prevent the template DNA from getting adsorbed to the surface of the reaction tube and also stabilizes polymerase activity.

The mechanisms behind the effects of some additives and contaminants are unclear. Less than 1% DMSO may affect the T_m of primers, the thermal activity of Taq polymerase and/or the degree of product strand separation. Higher DMSO concentration (10–20%) inhibits Taq polymerase activity from 50% to 90%. Ethanol does not affect activity up to concentrations of 10%.

How Can You Minimize the Frequency of Template Contamination?

Since the power of amplification is so great, the fear of getting a false positive is common (Dieffenbach and Dveksler, 1995). Here is a list of general PCR practices to minimize cross-contamination.

- Wear a clean lab coat and gloves when preparing samples for PCR.
- Have separate areas for sample preparation, PCR reaction setup, PCR amplification, and analysis of PCR products.

Table 11.7 Effects of Additives on Taq DNA Polymerase

Chemicals	Mode of Action	Amount for Enhancement or Inhibition
Ethanol		Slight enhancement at 10%
Urea	Lower target T_m for annealing.	Slight enhancement at 1–1.5M, but inhibition at greater than 2M
DMSO	Lower target T_m for annealing.	Enhancement at 1–10% (v/v) (www.alkami.com)
DMF	Lower target T_m for annealing.	12–15% (v/v) (Baskaran et al., 1996) ^a
Formamide	Lower target T_m for annealing. Increase specificity and yield by changing T_m of primer-template hybridization and lower heat destruction of enzyme.	Inhibition at 10% or greater
SDS	Prevent aggregation of enzyme.	Enhancement at 1.25–10% (v/v); Inhibition at 15% or greater
Glycerol	Enhance specificity by changing T_m . Extends Taq polymerase resistance to heat damage.	Inhibition at 0.01% or greater
Perfect match polymerase enhancer (PMPE) (Stratagene Inc.)		Enhancement at 5–20% (v/v) (www.alkami.com)
Ficoll 400 (Wittwer and Garling, 1991)		Approximately 1%
Gelatin		The optimal amount must be empirically determined.
Tween 20/NP40		100 µg/ml or 0.01–0.1% (w/v). 0.1–0.05% (v/v) Tween 20 0.05% (v/v) NP40
T4 Gene 32 protein (Schwarz et al., 1990)	Increase specificity and yield by changing T_m of primer-template hybridization.	0.05–0.1 nmole/amplification reaction (note: original publication incorrectly states 0.5–1.0 nmole)
Triton X-100	Prevents enzyme from aggregating.	0.01% (v/v)
Bovine Serum Albumin (BSA)	Neutralizes many factors found in tissue samples which can inhibit PCR.	10–100 µg/ml
Betaine		0.5–2.0M (Roche Molecular Biochemicals Web site) (1.8–2.5M) (Baskaran et al., 1996) ^a
Tetramethyl ammonium chloride (TMAC)		10–100 µm
PEG 6000		5–15% (w/v)
Spermidine	Reduces nonspecific reaction between polymerase and template DNA.	

Other references: For Taq DNA polymerase, Gelfand (1992, pp. 6–16); for the polymerase chain reaction, Coen (1995).

^aBaskaran et al. (1996) claims that combination of DMSO (5–10%) and betaine (1.1–1.4M) produces best results.

- Open PCR tube containing amplification products carefully, preferably in a room other than where the PCR reactions take place. Spin tubes briefly before opening a lid.
- Use screw cap microfuge tubes for templates and positive controls to control microaerosolization when opening tubes.
- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Discard pipette tips in a sealed container to prevent airborne contamination.
- Periodically clean lab benches and equipment with 10% bleach solution.
- Prevent contamination by using uracil-N-glycosylase (UNG) which acts on single- and double-stranded dU-containing DNA and destroys the PCR products (Longo, Berninger, and Hartley, 1990).
- Aliquot reagents, sterile water, primers, and other material into tubes to reduce the risk of contamination.
- When possible, avoid using plasmid DNA as a control. The DNA can contaminate the lab like a virus if not handled carefully. A safer control is a sample containing the target at high or low levels. Another method involves a synthetic oligonucleotide template that contains the sequence complimentary to primer binding region plus part of the sequence being amplified by the forward and reverse primers designed just for the initial testing of primers. They have major internal sequence deletions; thus they only serve to validate the primers. They are not amplified simultaneously with the test samples. If you must use plasmid DNA as a control, refer to the Appendix A for preparation of a plasmid DNA control solution that can be stored over a long period of time.

What Makes for Good Positive and Negative Amplification Controls?

The inclusion of reliable positive and negative controls in all your experiments will save time and eliminate headaches. Examples follow:

- Positive controls: Samples containing the target sequence at high copy number.
- Negative controls: One primer only, no Mg^{2+} , no enzyme, sample known to lack the target sequence, no RT step for RT-PCR.

Unfortunately, the above controls can also fail. Most often the failure originates in the preparation of the positive and negative

controls. Plasmid DNA is unstable at low concentrations during storage, especially in plain water or TE (10 Tris, 1 mM EDTA, pH 7). At dilute concentration, DNA can be lost by adsorption to the inner wall of a tube or be degraded by nuclease activity. A good way to store plasmid DNA (or control cDNA or genomic DNA) is in TE with 20 $\mu\text{g}/\text{ml}$ glycogen (molecular biology grade, nuclease free) in small aliquots in a -20°C freezer. Repeated freeze–thawing of control DNA should be avoided. The water used for any aspect of a PCR reaction should also be nuclease free, and stored in small volumes. Don't use a bottle of water that's been sitting in the lab for months. Microorganisms are too easily introduced.

What Makes for a Reliable Control for Gene Expression?

Good endogenous controls are constitutively expressed and change minimally while the target gene expression may vary greatly. Poor controls change their expression levels during the treatment, thus masking the target gene expression fluctuation. Bonini and Hofmann (1991) and Spanakis (1993) provide examples where inappropriate controls prevented the detection of biologically significant changes in gene expression. Some popular endogenous controls such as β -actin and glyceraldehyde dehydrogenase (GAPDH) are well known for having pseudogenes, and related genes, adding complexity to interpretation of results (Multimer et al., 1998; Raff et al., 1997). rRNA (28S, 18S, 5.8S, etc.) seems to be more constant in its level than other mRNA type housekeeping genes such as β -actin. Without a housekeeping gene that stays relatively constant (nothing really stays absolutely constant), a subtle change in gene expression will go undetected in the noise, and incorrect conclusions will result. The true level of a control should be monitored rather than taken for granted.

How Do the Different Cycling Parameters Affect a PCR Reaction?

The objective of the information in Table 11.8 is to provide guidelines to help you fine-tune a reaction based on your experimental observations. The data refer to Taq polymerase, but the trends hold true for most thermostable DNA polymerases.

Instrumentation: By What Criteria Could You Evaluate a Thermocycler?

Since the discovery of thermostable Taq DNA polymerase, numerous instrument companies have developed PCR cyclers, not

Table 11.8 Effect of Cycling Parameters on PCR

Stages of PCR	Standard Segment Time and Temperature	Below Optimum Duration	Above Optimum Duration
Initial denaturation	1–3 min 94°C (95°C for higher (55–60%) GC content)	Lower yield or no products Some genomic DNA needs more time, while PCR products or plasmid DNA need less time	Lower yield from premature loss of enzyme activity
Denaturation during cycling	5–20 s	Lower yield	Lower yield
Primer annealing	5–20 s 45–60°C Higher temperatures for more specific annealing	Lower yield	Nonspecific product formation
Primer extension	10–20 s 70–75°C	Lower yield	Lower yield
Cycle number	25–40	Lower yield	Increased error rate Nonspecific product formation
Final extension	1–2 min 70–75°C	Incomplete double-stranded DNA	Nonspecific product formation

only for amplification but for detection and analysis as well. A review of your current and anticipated needs will help you select the most appropriate machine within your budget.

Temperature Regulation

Consistent, predictable ramp times (the time required to transition from one temperature to the next) are crucial to achieve the desired PCR results. The time required to reach the 55°C annealing temperature from the 94°C denaturation temperature can vary one minute or more, depending on the cycler design. The consistency of the heating or cooling profile of samples can also vary with the instrument and introduce errors. If your goal is to run both tubes and plates, make sure that the tube fits the well snugly, as ill-fit tubes do not transfer heat well.

Programming Capability

If you run different cycling parameters, the capacity to link pre-existing programs rather than repeatedly installing old programs will save significant time. The ability to store many programs is also useful if you run many programs routinely or share a cycler with multiple users.

Minimum Manipulations

If your objective requires high-throughput analysis, it is recommended to use a cycler that combines amplification and analysis without further manipulation, such as gel electrophoresis or blotting. These postamplification processes require pipetting, opening and closing of reaction tubes, and so forth, which greatly increase the chance of contamination of other samples throughout the lab as the product contains enormous copies of the target sequence.

Reaction Vessels

Will your planned and unforeseen research require reactions in 0.2 ml, 0.5 ml tubes, or multiwell dishes? The ability to accommodate multiple sample formats usually pays off in the long run.

How Can Sample Preparation Affect Your Results?

Sample preparation can make the difference between good yield and no amplification. The purpose of sample preparation is to eliminate PCR inhibitors as well as to provide the DNA sequence available for PCR reaction. Compounds that inhibit PCR may co-purify with the DNA template and make PCR impossible (Reiss et al., 1995; Yedidag et al., 1996). Inhibitors do not have to be diffusible. Sometimes crosslinking of protein to DNA via carbohydrate groups can cause inhibition (Poinar et al., 1998). Addition of adjuncts such as bovine serum albumin (BSA) or T4 gene 32 protein can sometimes reverse the inhibition (Kreider, 1996). However, it is easiest to remove these inhibitors during the sample preparation than to figure how to reduce the degree of inhibition later. The qualities of good sample preparation follow:

- *Intact:* Undegraded and unnicked. DNA might appear intact immediately after isolation, but repeated use can result in nuclease-mediated degradation. This may result from incomplete removal of nucleases during the initial sample preparation or contamination of the sample during repeated usage; RNA requires a storage pH below 8.0 and special care to avoid RNase contamination.
- *Fixed:* DNA isolated from paraffin-embedded tissue sections and archived fixed tissues may pose problems due to nicking of DNA during tissue preparation. (Note: Human genome haploid equivalent is approximately 3 billion base pairs. Given that the distance between base pair is about $3.4 \text{ \AA}$, each human cell contains about 2 meters of DNA! A typical DNA isolation method shears genomic DNA in the process.)

- *Inhibitor-free*: Heparin, porpholin, SDS (<0.01%), sarkosyl, heme (Alkane et al., 1994), EDTA, sodium citrate, humic acid (Zhou et al., 1996), phenol, chloroform, xylene cyanol (Alkami PCR manual), and some heavy metals can inhibit PCR.
- *Clean*: $A_{260:280}$ ratio of 1.8 to 2.0; Free of protein and carbohydrate. (See Chapter 4, “How To Properly Use and Maintain Laboratory Equipment,” for situations where $A_{260:280}$ ratios prove unreliable.)
- *RNA*: Free of DNA.

How Can You Distinguish between an Inhibitor Carried over with the Template and Modification of the DNA Template?

If it is diffusible inhibition of a thermostable DNA polymerase, adding the sample in smaller quantity lessens the effect whereas the effect worsens with more sample. If the problem is caused by template modification, dilution will have no effect. Compounds such as *N*-phenacylthiazolium bromide (PTB) may eliminate inhibition (Poinar et al., 1998) caused by agents crosslinking to the template. PCR inhibitors can be detected by performing reactions in the presence of commercially available exogenous internal positive controls, which can be added to your PCR reaction without hampering the amplification of your target.

What Are the Steps to Good Primer Design?

Step 1. Consider the Objectives

What must the PCR accomplish? What pressures does this put on the primers?

- Must you identify few or many targets? The identification of several targets requires numerous primers, increasing the difficulty of avoiding 3' overlaps.
- Must you clone the full-length coding region of a gene? For long PCR, you may use the nearest-neighbor algorithm for selection of T_m (Rychlik et al., 1990).
- Must you generate quantitative data? PCR efficiency becomes more critical, as does avoiding primer-dimers.
- Must you design primers without knowing the exact sequence of the specific species based on information from another species (i.e., design primers for the rat gene X using mouse or human gene sequence for gene X)? If so, aligning as many sequences of gene X from as many organisms as you can collect in order to select the most conserved region for primer design increases the likelihood of success.

- Must you avoid amplifying pseudogenes? What is known about pseudogenes to your target? A preliminary review of the research literature can save you time and headaches. Unfortunately, there are more pseudogenes than are reported. One quick way to search for pseudogene amplification with your selected primer pairs is to do a BLAST search (see Appendix C). However, the only sure way to avoid pseudogenes is to design primers across exon–exon junctions and test for them at the bench by amplifying genomic DNA. Processed pseudogenes do not have introns, so they can be amplified when the PCR primer extend over the two exon junctions.

- Are you searching for a single nucleotide polymorphism (SNP)? SNP primer design requires specialized strategies (Kwok et al., 1995; Wu et al., 1991).

- Must you design a small amplicon to increase detection of the gene in samples where the chance of amplifying a long sequence is unlikely (i.e., paraffin embedded sections, forensic samples, and partially degraded samples)?

Step 2. Apply the Sequence Analysis Programs to Develop Candidate Primers

These programs are described in Appendix B.

Step 3. Apply Good Primer Design

Refer to the generally accepted elements of good primer design (Dieffenbach and Dveksler, 1995). The new nearest-neighbor model based on DNA thermodynamics data for PCR primer design is also recommended (SantaLucia, 1998).

- The optimum length of primers for use with Taq DNA polymerase is between 18 and 28 bases for specificity (This number may vary with enzymes with greater heat stability.) The longer primer gives more specificity but tends to anneal with lower efficiency and results in a significant decrease in yield. A good pair of primers has melting temperature (T_m) 55°C to 60°C. Shorter primers (less than 15 nucleotide long) anneal very efficiently, but they may not give sufficient specificity. Longer primers may be useful when distinguishing multiple gene forms sharing a high degree of sequence homology. The probability of finding a match using a set of 20 nucleotide long primers is $(\frac{1}{4})^{(20+20)} = 9 \times 10^{-26}$ (Cha and Thilly, 1995). It is likely that this set of primers will amplify another gene in the mammalian genome (3×10^9 bp per haploid genome).

- GC content should be between 40% and 60%. The T_m of both primers should be similar to each other and similar to the primer-binding sites at the ends of the fragment to be amplified to achieve an optimal annealing temperature and amplification.

- 3'-end complementarity between primers and self-complementarity within primers must be avoided because it may increase primer-dimer formation and reduce PCR efficiency. This is more problematic when you have a low number of target gene copies.

- Avoid runs of G/C, especially guanidine.

- When performing RT-PCR, design primers to go across exon-exon junctions to avoid amplifying genomic DNA. Since the use of DNase has a negative effect on RNA, it is better to avoid genomic DNA amplification by primer design (Huang, Fasco, and Kaminsky, 1996).

- Include controls lacking RT unless you have shown that this set of primers does not amplify genomic DNA.

- After designing the primers, search for specificity using BLAST (Basic Local Alignment Search Tool), a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA (Appendix C). This is especially important for those genes with many pseudogenes and related genes. If you are executing RT-PCR, this is essential. Even a trace amount of genomic DNA left in the RNA sample preparation can give sufficient amplification of those genes. Often these PCR products are indistinguishable by gel electrophoresis and make data interpretation difficult.

- You may add exogenous sequence to the 5' end of primers for cloning and other purposes.

- When sequence information is ambiguous, substitute deoxyinosine for the unknown nucleotide, and place the ambiguous sequence on the 5' end. Design and test different primers to determine which works best. Inosine is naturally found in some tRNA. It base pairs with A, C, and U in the translation process (Martin et al., 1985; Kwok et al., 1995).

- Before testing the primers with your test sample, measure the quantity of your primers, and then test with the positive controls. You cannot assume that all primers have the correct sequence. Inefficient desalting, incorrect labeling, and other quality control problems can ruin a primer's performance.

Step 4. Develop and Apply a Primer Testing Strategy

If your goal is to study many genes, then you may want to consider setting a standard thermal cycling condition to run all your PCR reactions, even though they won't produce the optimal PCR results. If your goal is to study a few genes, then the design is more straightforward.

This discussion about primer design is relevant to basic PCR. The bibliography provides references for primer design relevant to multiplex or nested PCR applications.

Which Detection and Analysis Strategy Best Meets Your Needs?

It is crucial that your strategy be consistent with your purpose. If you require quantitative data, a hybridization-based strategy is not ideal. Probe labeling, membrane transfer, and hybridization conditions can introduce variability. If resolution is crucial to your study, PAGE rather than agarose electrophoresis might be required. Because the potential for variability usually increases with the number of manipulations required to generate the data, real-time PCR usually provides greater reproducibility along with time savings.

Table 11.9 compares commonly applied detection methods.

TROUBLESHOOTING

Even the most thorough, insightful planning cannot guarantee success, and PCR can generate results indicative of complete failure or a reaction in need of optimization. The troubleshooting section is organized to reflect the fact that any given PCR problem can have several underlying explanations. The optimization of cycling conditions, primer concentration, and other parameters discussed throughout the chapter can also help resolve a problem.

No Product

Template

Is the target sequence absent?

Amplify housekeeping gene or some gene you know is present as a control; perform standard curve assay with plasmid or amplicon to estimate the dynamic range of detection. This range also indicates the lower limit of detection.

Table 11.9 Comparison of Different Detection Strategies

	Method	Indicates Size	Quantitative	High-throughput	Specificity	Reproducibility
Post PCR	Agarose gel electrophoresis					
	Intact PCR product	Yes	Poor	Poor	Poor	Poor
	Restriction enzyme analysis	Yes	Fair	Poor	Fair	Fair, if the PCR reaction falls in the exponential phase
	Blots/hybridization	Yes	Poor	Poor	Fair	Poor
	Scanning/densitometer	Yes	Fair	Poor	Fair	Poor; too many variables
	Nested PCR	Yes	Fair	Poor	Good	Fair
	PAGE electrophoresis	Yes	NA	Fair	Excellent	Poor
Real time	(sequencing gel)					
	Automated detection and analysis systems	No	Excellent	Excellent	Excellent	Excellent

Enzyme

Is the enzyme inactive?

Did positive controls work?

Primer

Is the primer poorly designed?

Utilize several different amplicon locations to design the primers to increase your chance of success.

Cycling Parameters

Was there insufficient amplification?

Take a portion of the PCR products and amplify further or repeat with a larger quantity of starting material or test with nested PCR.

Lower or raise annealing temperature (See Tables 11.3 and 11.8).

Buffer

Were one or more buffer components faulty?

Include a positive control such as a commercially tested endogenous control, or a pretested set of reagents

Mg²⁺ concentration is not optimum?

Raise or lower the concentration as per Table 11.5.

Other

Was the detection method sufficiently sensitive?

Prepare a standard curve with a positive control to determine the detection limit.

Smear on the Gel

Template

Was the template copy number too large?

Was the template degraded?

Enzyme

Was too much enzyme and/or too much template included?

Primer

Is the primer design following the design guideline? Is the concentration of primer too low or too high? Do primers lack specificity?

Cycling Parameters

Too many amplification cycles?
Is the annealing temperature too low?

Buffer

Is the Mg^{2+} concentration optimal?

Lower the concentration as per Table 11.5.

Other

Was the appropriate electrophoresis buffer and/or gel concentration used?

Wrong Product

Template

Is the template copy number too large or is the template DNA degraded?

Test a negative control sample to determine if data represent an artifact.

Enzyme

Use a hot-start strategy (Ehrlich, Gelfand, and Sninsky, 1991) to increase specificity.

Primer

Inappropriate primer design?

Apply nested PCR, sequencing, restriction analysis or hybridization to troubleshoot.

Perform a BLAST search to assess possibility of amplifying a different gene (Appendix C).

Cycling Parameters

Too many amplification cycles?
Annealing temperature too low?

Buffer

Is the Mg^{2+} concentration optimal?

Lower the concentration as per Table 11.5.

Other

Was the appropriate electrophoresis buffer and/or gel concentration used?

Faint Band of the Correct Size/Low Yield

Template

Poor quality DNA or RNA?

Check the sample for degradation, inhibitor, or contamination.

Enzyme

Use a hot-start strategy to increase specificity.

Primer

Examine primer design for unmatched T_m of the forward and reverse primers, runs of pyrimidine and purine, or other unfavorable sequence; if a primer-dimer band (lower molecular weight) is visible, a hot-start strategy might increase the yield of the desired product.

Cycling Parameters

Insufficient amplification cycles?

Continue amplification with fresh reagents

Annealing temperature not optimum?

Increase/decrease for more yields.

Buffer

Nonoptimal Mg^{2+} concentration?

Increase concentration as per Table 11.5.

Positive Control Generated Product, but Your Sample Did Not

Template

The sample did not contain the target sequence at detectable level.

Pipetting problem? DNA sample never added to the reaction?
It is always a good idea to do two to four reactions to exclude such a possibility.

Enzyme

Low specificity and yield?

Use modified form of Taq DNA polymerase such as TaqGold™ to increase both specificity and yield. This enzyme is inactive until thermal activation to provide a hot-start for increased specificity. At the same time this enzyme is time-released, providing more enzyme in the later cycles when more enzyme increases yield. Decreased mispriming also increases the amount of the desired PCR products (Abramson, 1999).

Primer

Primer design not optimal if primer-dimer is formed?

Redesign.

Cycling Parameters

Insufficient amplification cycles?

Continue amplification with fresh reagents.

If the yield of the positive control is also low, optimize annealing and denaturation temperature and duration of each hold time to increase yield.

Buffer

Mg²⁺ concentration is not optimal?

Increase concentration as per Table 11.5.

Other

Presence of PCR inhibitors?

Test an exogenous IPC (internal positive control) for troubleshooting, or do mixing experiment to test if addition of your sample inhibits the positive control.

Is an inhibitor crosslinked to the DNA template?

Try adding adjunct such as PTB

The troubleshooting discussion above further illustrates how appropriate controls can simplify or eliminate much of the troubleshooting effort. Prevention is the key.

Misincorporations of Nucleotides

Template

Too much single-stranded DNA sample due to insufficient extension time or not having enough quantity of one of the primers?

Enzyme

Too many units of DNA polymerase present?

Primer

Nonoptimal T_m causes pre-PCR annealing to secondary, unintended sites?

Check sequence for hot spots for mispriming.

Cycling parameters

Ramp time too long?
Annealing temperature too low?

Buffer

Mg^{2+} concentration too low?

Check dNTP and template concentration.
Adjust as per Table 11.5.

RT-PCR

Despite the increased interest in RT-PCR, this technique can be more challenging than DNA PCR in many ways. Here are some parameters to keep in mind:

- Isolation and purification of RNA requires greater care.
- Design of primers spanning a large intron may be necessary to avoid amplifying contaminating genomic DNA.
- DNase treatment of RNA preparation may affect different genes differentially for the subsequent PCR (Huang, Fasco, and Kaminsky, 1996); thus use it only as the last resort. Residual DNase I can reduce the yield of PCR products.
- The most frequently used reverse transcriptases are MuLV, *rTth* DNA polymerase, and SuperScript™ (Life Technologies). For RNA with excessive secondary structure or high GC content, apply *rTth* DNA polymerase. Its greater heat stability allows for higher reaction temperatures using a gene-specific reverse primer,

which increases specificity of the RT-PCR reaction. However, these conditions may increase hydrolysis of RNA.

- The choice of primers for the cDNA synthesis includes random primers (nonamers and hexamers), oligo dT and gene-specific primers. For cloning full-length gene, use oligo dT. Use random hexamers for multiplex or when the test sample may not be of good quality (i.e., clinical samples), where full-length mRNA is difficult to obtain (i.e., paraffin-embedded tissue), and where the position of the amplicon is distant from the poly (A) tail. The latter case is especially important when RNA secondary structure prevents full-length synthesis of the first-strand cDNA via the relatively low temperature (37–42°C) RT reaction. Therefore your choice of primers for RT depends on the relative distance between the priming site, the amplicon location and the gene structure. You may want to avoid oligo dT if the following conditions apply to your gene:

- Presence of long 3'-untranslated region (UTR) (>1 Kb) or the length of it is unknown.

- The amplicon site is at the 5' end of a long transcript.

- The amplicon site is at the 5' end of a GC-rich gene.

SUMMARY

This chapter has discussed basic PCR technology issues. The complexity of more advanced techniques such as allele-specific amplification, long PCR, RACE, DICE, competitive RT-PCR, touchdown, multiplex PCR, nested PCR, QPCR, and in situ PCR could not be covered in this review.

The intellectual and biochemical strategies discussed within this chapter were not designed to answer every question related to PCR, but to provide a foundation to help you, better ask and answer questions that you will encounter. Combined with the resources provided within this chapter, the author hopes this chapter provides you with new insight to evaluate and meet your PCR needs.

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APPENDIX A

PREPARATION OF PLASMID DNA FOR USE AS PCR CONTROLS IN MULTIPLE EXPERIMENTS

Have you ever failed to amplify a section of a plasmid that previously produced the desired PCR product? Your problem is not unique. Often plasmid DNA at low concentration of DNA is degraded by nuclease or adsorbs to the wall of the plastic tube during storage and handling. This protocol produces plasmid DNA that is stable for months and years if stored at -20°C and generates reproducible standard curves.

The addition of glycogen ($20\text{ }\mu\text{g/ml}$ final concentration) in 10 mM Tris, 1 mM EDT (pH8.0) buffer can protect DNA from degradation by nuclease as well as loss from adsorption to the tube. After making serial ten-fold dilutions ($100\text{ }\mu\text{l}$ of DNA in $900\text{ }\mu\text{l}$ of TE), aliquot the solution in $100\text{ }\mu\text{l}$ or less volume and store at -20°C . For preparation of TE buffer, use fresh nuclease-free water. "Sterile" water sitting on the lab bench for one week or more may contain contaminants as well as nucleases.

APPENDIX B

COMPUTER SOFTWARE FOR SELECTING PRIMERS*

Primer v. 1.4 (DOS)
PINCERS (Macintosh)
Oligonucleotide Selection Program (Macintosh, DOS, Digital VAX/VMS, SUN SPARC-based workstations)
Right Primer: Primer Design Utility
Gene Runner 3.0
Oligo 5.0 (DOS)
Oligo 4.0
DNASIS 2.0 (Windows)
MacDNASIS (Macintosh)
GeneWorks (Macintosh)
Lasergene (DOS, Windows, Macintosh)
Eugene™ (DOS)
GeneJockey (Macintosh)
Wisconsin Sequence Analysis Package (Digital VAX/VMS, IBM RS6000, Sun SPARC-based workstations, Silicon Graphics Workstation)
MacVector (Macintosh)
PRIMER PRIMER (Macintosh, DOS, PowerMac)
DesignerPCR (Macintosh)
Vector NT1 (Windows, Macintosh)
Primer Designer (Macintosh, DOS)
Primer Express™
HYTHER (PC-Windows, UNIX and Web-based platforms) available for license at <http://jsll.chem.wayne.edu/Hyther/hythermenu.html>.

*Data from Dieffenbach and Dveksler (1995).

APPENDIX C

BLAST SEARCHES

There are many genes that share local sequence homology with a primer 18 to 30 nucleotide long. For example, the beta-actin primer shares 100% homology with pseudogenes, gamma-actin, and related genes. It is therefore misleading to use this primer to estimate the level of beta-actin gene expression. Some of the PCR products will be derived from these genes, but you have no way to tell how much came from the true beta-actin gene. Pseudogenes are not translated into protein and have no biological significance, so your RT-PCR result may not relate to immunological data or biochemical assays. In some cases it will amplify other genes not even related to the one you are investigating. For this reason it helps to know the BLAST search information before ordering primers.

The BLAST programs (<http://www.ncbi.nlm.nih.gov/BLAST>) have been designed for speed, with a minimal sacrifice of sensitivity to distant sequence relationships. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random background hits. BLAST uses a heuristic algorithm that seeks local as opposed to global alignments, and it is therefore able to detect relationships among sequences that share only isolated regions of similarity (Altschul et al., 1997). For a better understanding of BLAST, refer to the BLAST instructional course, which explains the basics of the BLAST algorithm.

APPENDIX D

USEFUL WEB SITES

Topics	Content and URL
Basic PCR information	Weizmann Institute of Science Genome and Bioinformatics site http://bioinformatics.weizmann.ac.il/mb/bioguide/pcr/contents.html
Collection of PCR protocols	Standard PCR protocols http://www.protocol-online.net/molbio/PCR/standard_pcr.htm
Optimization of PCR	Primer design and reaction optimisation. E. Rybicki, Department of Microbiology, University of Cape Town. In <i>Molecular Biology Techniques Manual: Third Molecular Biology Techniques Manual</i> , V. E. Coyne et al., eds. http://www.uct.ac.za/microbiology/pcroptim.htm
Standard PCR guideline	PCR Primer: Strategies to improve results provided by G. Afseth of Perkin Elmer at Northwestern University (1997) http://www.biotechlab.nwu.edu/pe/index.html
Molecular biology methods	Current Protocols in Molecular Biology http://www.wiley.com/cp/cpmb/mb0317.htm Elsevier Trends Journals Technical Tips online http://research.bmn.com/tto Molecular biology reagents and procedures. Dartmouth University http://www.dartmouth.edu/artsci/bio/ambros/protocols

APPENDIX D (Continued)

PCR protocols and online manual	Alkami Quick Guide™ for PCR. A laboratory reference for the polymerase chain reaction. 1999 http://www.alkami.com/qguide/idxguide.htm
PCR protocol	Roche Molecular Biochemicals PCR protocol http://206.53.227.20/prod_inf/manuals/pcr_man/index.htm
Links to many sources of basic PCR and other molecular biology information	ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) Molecular Biology Server http://www.expasy.ch/
Multiplex PCR	Multiplex PCR: Critical parameters and step-by step protocol. O. Hehegariu et al., <i>Biotech.</i> 23(1997):504–511 http://info.med.yale.edu/genetics/ward/tavi/bt/BT(23)504.pdf
Various PCR topics, including multiplex PCR	Tavi's PCR site (Octavian Henegariu) on variety of topics http://info.med.yale.edu/genetics/ward/tavi/PCR.html
Primers	Primers! Web site http://www.alkami.com/cntprmr.cgi?url=http://www.williamstone.com/primers/javascript/ Hyther http://jsll.chem.wayne.edu/Hyther/hythermenu.html
PCR chat room	Protocol online (discussion) http://www.protocol-online.net/discussion/index.htm
Real-time PCR	References for TaqMan real-time assay http://www.appliedbiosystems.com/ab/about/pcr/sds/taqrefs.html
Gene quantitation	References on absolute and relative gene quantitation by PE Biosystmes http://www.appliedbiosystems.com/ab/about/pcr/sds/taqrefs.html#rev
Gene search and validation BLAST	BLAST (National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) family of programs http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=0

Caution: The dynamic nature of the Web allows us to provide more up-to-date information. However, there are major challenges associated with information available on the Web. Some of the major challenges are as follows: (1) Since it is easier for anyone to publish on the Web, its content may not be evaluated nor accurate. (2) The URL address as well as its content may change or even disappear without notice, thus quickly invalidating any list of “useful” sites. All of the Web sites given in this section were selected to give the reader sources of information only and by no means recommended as “valid” source. It is up to the users to determine what is useful. The author highly recommends that readers use their own judgment before adapting any information given.